UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

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OFFICE OF
PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: Dicamba: Structural Chromosome Aberration Study in Chinese Hamster Ovary (CHO) Cells. Caswell No. 295.

TO: Stubbs/Asbury, PM-41
Registration Division (TS-767C)

FROM: K. Clark Swentzel K. Clark Swentzel, 11/30/87
Section III
Toxicology Branch (TS-769C)

THRU: Marcia van Gemert, Ph.D. M. van Gemert 11/30/87
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and

Theodore Farber, Ph.D.
Chief, Toxicology Branch (TS-769C)

The attached Data Evaluation Report was prepared for a mutagenicity study entitled: (Dicamba) Chromosome Aberrations in Chinese Hamster Ovary (CHO) Cells. Although Dicamba did not induce chromosome aberrations in this study, the registrant did not provide the purity or stability of the test material. Therefore, the classification of this study is unacceptable. This study can be upgraded if the purity and stability of Dicamba, under experimental conditions, as well as solubility data, to support the highest concentration selected for assay, are provided.

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Reviewed by: K. Clark Swentzel
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DATA EVALUATION REPORT

STUDY TYPE: Mutagenicity: in vitro chromosome abertrations CASWELL NO.: 295

EPA ID NO.:

TEST MATERIAL: 3,6-dichloro-o-anisic acid

SYNONYMS: Dicamba, Banvel

REPORT NO.: T5245.337

SPONSOR: Sandoz Crop Protection Corp.

TESTING FACILITY: Microbiolological Associates,Inc.

TITLE OF REPORT: (Dicamba) Chromosome Aberrations in Chinese Hamster Ovary (CHO) Cells

AUTHOR: Donald L. Putman

REPORT ISSUED: April 7, 1987

Conclusions

Technical Dicamba was tested in the chromosome aberration assay, using cultured Chinese hamster ovary cells, both in the presence and absence of an Aroclor induced S-9 activation system at stated concentrations of 2330, 1170, 590 and 300 ug/ml. No significant increase in structural chromosome aberrations was reported or evident from the submitted data under either test condition. Therefore, technical Dicamba was negative in the CHO chromosome aberration assay under the conditions of this test.

Core-classification: Unacceptable: may be upgraded if the purity and the stability data, under assay conditions, of the evaluated Dicamba are provided, with solubility data to support the highest concentration selected for assay.

Quality assurance: A statement was submitted with the study.

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Materials:

Test material

Technical Dicamba (Lot# 52625110), described as tan flakes: expiration date--May, 1996. Dissolved in DMSO to prepare test solution.

Positive control

S-9 activation: Cyclophosphamide (CP), lot 85F-0054; dissolved in distilled water at a working concentration of 10 mg/ml.

Non-activation: Triethylenemelamine (TEM), lot 34898; dissolved in distilled water to prepare a working concentration of 100 ug/ml.

Cell line: Chinese hamster ovary (CHO-K_1) cells (repository number CCL 61, American Type Culture Collection, Rockville, MD).

Methods (Detailed description provided on appended pages 1-3)

S-9 Fraction: The S-9 fraction was prepared from the livers of adult male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254. The S-9 was mixed with cofactor to contain 15 ul S-9, 1.4 mg NADP and 2.7 mg isocitric acid per ml growth medium with 2.5% serum.

Preliminary cytotoxicity study

Two cytotoxicity studies were performed in an attempt to achieve a cytoxtoxic concentration of Dicamba. Cell cultures , seeded at $5x10^5$ cells/25cm^2 flask, were exposed to Dicamba concentrations of 0.1 to [redacted] ug/ml in the first study (appended pages 4 and 6) and 740 to 2560 ug/ml in the second study (appended pages 5 and 7) with and without S-9 activation. The cells were also exposed to test material solvent (DMSO), but not to either positive control. The objective of this study was to determine the dosage level of test material that inhibited cell growth and/or induced cell cycle delay. Treatment was carried out by refeeding the flasks with 5 ml complete medium for the non-activation study or with 5 ml S-9 reaction mixture for the activation study, to which 50 ul of the test solution or solvent alone was added. The cells were treated for 6 hours in the non-activated system; two hours after initiation of treatment, 50 ul of 1mM BrdU was added to each flask and incubation continued as required. In the activation system, the cells were treated for two hours after which time the treatment medium was removed, the cells washed with PBS, refed with 5 ml complete medium containing 0.01 mM BrdU and returned to the incubator for a total of 24 hours from BrdU treatment. Following incubation and treatment, the cells were harvested by trypsinization and counted to estimate relative cell growth. Metaphase preparations were stained for sister chromatid differentiation using a modified fluorescence plus giemsa technique (Perry and Wolff, 1974). The investigator evaluated the slides for the percentage of first, second and third division metaphase cells for estimation of the test material effect on cell cycle kinetics.

Cytogenetics assay

The CHO cells were seeded at approximately $5x10^5$ cells/25 cm^2 flask and incubated

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at 37°C in a humidified atmosphere of 5% CO_2 in air for 18-24 hours for this assay.

S-9 activation

Duplicate flasks were refed with 5ml S-9 reaction mixture to which the test, control or solvent doses were added. The cells were exposed for 2 hours incubation after which time the treatment medium was removed. The cells were washed with PBS, refed with complete medium and incubated an additional 6 hours before Colcemid was added at a final concentration of 0.1 ug/ml.

Non-activation

Duplicate flasks were refed with 5ml complete medium before the appropriate exposure. The cells were exposed for at least 8 hours incubation. Two hours prior to the scheduled cell harvest, the treatment medium was removed in order to avoid interference with cell collection and fixation; the cells were washed with PBS and refed growth medium containing 0.1 ug/ml Colcemid.

Untreated cells were included as a control for both the activated and non-activated assays.

Exposure concentrations

The nominal concentrations of Dicamba chosen for the assay were 2560, 1280, 640 and 320 ug/ml. However, during the cytotoxicity study, it was discovered that 1000 ug/ml decreased the pH of the assay mixture so adjustments with 1N NaOH were necessary. Due to dilution with NaOH, the actual concentrations of Dicamba attained in the assay were 2330, 1170, 590 and 300 ug/ml.

Assay concentrations of CP and TEM were 100 and 1 ug/ml, respectively.

Metaphase analysis

Two hours after the addition of Colcemid, metaphase cells were harvested for both the activated and non-activated studies by mitotic shake-off.

One hundred cells per treatment group were scored. The investigator evaluated the cells for chromatid gaps and breaks, chromosome gaps and breaks, chromatid fragments, acentric fragments, dicentrics, rings, triradials, quadriradials, complex rearrangements, pulverized chromosomes and cells and severely damaged cells (>10 aberrations).

Evaluation of data

The cytotoxic effects of treatment were expressed relative to the solvent-treated control (relative cell growth). Additional observations for each group included the numbers and types of aberrations, the percentage of structurally damaged cells in the total population of cells and the frequency of structural aberrations per cell. Statistical analysis of the frequency of the structural aberrations per cell was performed using Student's t-test. Although chromatid and chromosome gaps were scored, these data were not included in this analysis. The t-test was used to compare (pairwise) the number of aberrations per cell of each treatment group with that of the solvent control.

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Evaluation criteria

The number of cells with chromosome aberrations in the negative and solvent control could not be greater than 6%. The number of cells with chromosome aberrations in the positive control had to be significantly increased ($p<0.05$, Student's t-test) relative to the solvent control or the untreated control if a solvent other than water was used.

Results

Preliminary cytotoxicity study

The concentration range for Dicamba in this study was 0.1-2560 ug/ml. The investigator indicated that the highest concentration was determined by the limit of solubility, however, the registrant did not submit data to substantiate this. According to the report, there were no solubility problems with the concentrations used in this study.

The submitted data (appended pages 4-7) showed that Dicamba did not induce cytotoxicity in CHO cells at the highest concentration tested. Values for relative cell growth and cell cycle kinetics were comparable between cells treated with Dicamba and DMSO, both in the presence and absence of S-9 activation.

Cytogenetic assay

Data representing the cytogenetic analysis of CHO cells treated with Dicamba in the presence and absence of S-9 activation are provided on appended pages 8 and 9, respectively. According to the investigator's statistical analyses, these data indicate that the frequency of cells with structural aberrations was not significantly increased above that of the solvent control ($p>0.05$, Student's t-test) in both the S-9 activated and non-activated assays. The increased incidence of aberrations induced by the positive controls, 0.45 and 0.69 aberrations per cell from TEM and CP, respectively, were statistically significant ($p<0.01$, Student's t-test) in comparison to the untreated controls.

Conclusions

Based on the evaluation criteria described herein, the test material (technical Dicamba), did not induce structural or numerical chromosome aberrations in CHO cells in the presence or absence of an S-9 metabolic activation system, under the conditions of this assay.

Chromosome aberrations induced by the positive controls support the validity of the assay.

The registrant should provide the purity and stability, under experimental conditions, of the test material in order for this test to be acceptable. Also, solubility data, to support the highest concentration selected for assay, should be submitted.

Citation

Perry, P. and Wolff, S. 1974. New Giemsa Method for Differential Staining of Sister Chromatids. Nature 251: 156-158.

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MATERIALS AND METHODS

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Materials

Mammalian Cells:	Chinese hamster ovary (CHO-K_1) cells (repository number CCL 61, American Type Culture Collection, Rockville, MD)
Biological Reagents:	McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS), 100 units penicillin and 100 ug streptomycin/ml, and 2 mM L-glutamine (complete medium) Phosphate buffered saline (PBS) Trypsin, 0.1% in PBS Cofactor pool: 45 mg isocitric acid and 24 mg nicotinamide adenine dinucleotide phosphate (NADP) per ml in distilled water S-9 homogenate: 9000 x g supernatant of an Aroclor 1254 induced Sprague Dawley rat liver homogenate Colcemid, 10 ug/ml Potassium chloride (KCl), 0.075 M Carnoy's fixative (methanol: acetic acid, 3:1) Giemsa stain
Supplies:	Pipets, assorted sizes Plastic tissue culture flasks Centrifuge tubes Glass slides and coverslips
Chemicals:	Solvent for test article (DMSO) Triethylenemelamine (TEM) Cyclophosphamide (CP)

Methods

The S-9 was prepared according to established procedures. Adult male Sprague-Dawley rats, 200-300 gm, were induced by a single intraperitoneal injection of Aroclor 1254 at a dose of 500 mg/kg body weight five days prior to sacrifice. The animals were sacrificed and the livers aseptically removed. The excised tissue was rinsed three times in cold sterile 0.15 M KCl and then homogenized in a Polytron Tissuemizer at a concentration of 1:3 (w/v) in 0.15 M KCl. The supernatant fraction (S-9) was collected following centrifugation at 9000 x g for 10 minutes at 4±2°C, portioned into aliquots for daily use, and stored frozen at ≤-70°C until used. Each bulk preparation of S-9 was assayed for its ability to metabolize 2-aminoanthracene and 7,12 dimethylbenz(a)-anthracene to forms mutagenic to S. typhimurium TA100.

Immediately prior to use, the S-9 was mixed with the cofactor pool to contain 15 ul S-9, 1.4 mg NADP and 2.7 mg isocitric acid per ml growth medium with 2.5% serum.

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The toxicity test was performed for the purpose of selecting dose levels for the chromosome aberration assay and consisted of test article effect on cell growth potential and cell cycle delay. CHO cells were seeded for each treatment condition at approximately 5 x 10^5 cells/25 cm^2 flask and were incubated at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air for 16-24 hours. In the repeat toxicity test, the cells were treated 26 hours after seeding due to a delay caused by difficulty in neutralization of the test article stock solution; this deviation was documented in the raw data with a Deviation Report. Treatment was carried out by refeeding the flasks with 5 ml complete medium for the non-activation study or with 5 ml S-9 reaction mixture for the activation study to which was added 50 ul dosing solution of test article in solvent or solvent alone. The cells were treated for 6 hours in the non-activated system; two hours after initiation of treatment, 50 ul of 1mM BrdU was added to each flask and incubation continued as required. In the activation system, the cells were treated for two hours after which the treatment medium was removed, the cells washed with PBS, refed with 5 ml complete medium containing 0.01 mM BrdU and returned to the incubator for a total of 24 hours from BrdU treatment. The cells were harvested by trypsinization and counted for estimation of relative cell growth. Metaphase preparations were prepared and stained for sister chromatid differentiation using a modified fluorescence plus giemsa technique (Perry and Wolf, 1974). Slides were evaluated for the percentage of first, second and third-division metaphase cells for estimation of the test article effect on cell cycle kinetics.

For the chromosome aberration assay, CHO cells were seeded at approximately 5 x 10^5 cells/25 cm^2 flask and were incubated at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air for 18-24 hours. Treatment was carried out by refeeding duplicate flasks with 5 ml complete medium for the non-activation study or with 5 ml S-9 reaction mixture for the activated study to which was added 50 ul of dosing solution of test or control article in solvent or solvent alone. An untreated control consisting of cells in complete medium was also included.

In the non-activation study, the cells were exposed for 8 hours (or longer in the event of cell cycle delay) at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air. Two hours prior to the scheduled cell harvest, the treatment medium was removed in order to avoid interference with cell collection and fixation and the cells were washed with PBS and refed with growth medium containing 0.1 ug/ml of colcemid. In the S-9 activated study, the cells were exposed for 2 hours at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air. After the exposure period, the treatment medium was removed, the cells were washed with PBS, refed with complete medium and returned to the incubator for an additional 6 hours. At this time, colcemid was added to duplicate flasks for each treatment condition at a final concentration of 0.1 ug/ml.

Two hours after the addition of Colcemid, metaphase cells were harvested for both the activated and non-activated studies by mitotic shake-off. The cells were collected by centrifugation at approximately 800 rpm for 5 minutes. The cell pellet was resuspended in 2-4 ml 0.075 M KCl and allowed to stand at room temperature for 5 minutes. The cells were collected by centrifugation, the supernatant was aspirated and the cells were fixed with two washes with approximately 2 ml Carnoy's fixative and were stored overnight or longer in Carnoy's fixative at approximately 4°C.

To prepare slides, the fixed cells were centrifuged at approximately 250 x g for 5 minutes, the supernatant fluid decanted, and the cells were resuspended to opalescence in Carnoy's fixative. One-2 drops of cell suspension were dropped onto the center of a moist glass slide and allowed to air dry overnight. Slides were identified by the study number, date prepared and the treatment condition. The dried slides were stained with 5% Giemsa, air dried and permanently mounted.

Slides were coded with random numbers and scored without regard to treatment group. Fifty metaphase cells were scored in each duplicate treatment flask for a total of 100 cells per treatment group. Cells were evaluated for chromatid gaps and breaks, chromosome gaps and breaks, chromatid fragments, acentric fragments, dicentrics, rings, triradials, quadriradials, complex rearrangements, pulverized chromosome(s) and cells and severly damaged cells (>10 aberrations).

Controls

TEM was used as the positive control in the non-activated study at a concentration of 1 ug/ml. CP was used as the positive control in the S-9 activated study at a concentration of 100 ug/ml. The solvent vehicle for the test article was used as the solvent control at the same concentration as that found in the test article-treated groups. Growth medium was used in the untreated control.

Evaluation of Test Results

The cytotoxic effects of treatment are expressed relative to the solvent-treated control (relative cell growth). The number and types of aberrations found are presented for each treatment group. The percentage of structurally damaged cells in the total population of cells examined was calculated for each group. The frequency of structural aberrations per cell was also calculated and reported for each group. Chromatid and chromosome gaps are presented in the data but not included in the total percentage of cells with one or more aberrations or in the frequency of structural aberrations per cell. Statistical analysis of the frequency of structural aberrations per cell was performed using the Student's t test. The t test was used to compare pairwise the number of aberrations per cell of each treatment group with that of the solvent control.

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TABLE 1

PRELIMINARY TOXICITY TEST USING TECHNICAL DICAMBA IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

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Treatment[1]	Growth Potential		Cell Cycle Kinetics		
	Cells/flask ($\times 10^6$)	Relative Cell Growth[2] (%)	Percentage of Cells in M_1	M_2	M_3
DMSO	3.51	100	6	94	0
Technical Dicamba					
1000 ug/ml	3.65	104	10	90	0
300 ug/ml	3.77	107	12	88	0
100 ug/ml	3.77	107	8	92	0
30 ug/ml	3.45	98	4	96	0
10 ug/ml	3.92	112	5	95	0
3 ug/ml	3.30	94	9	91	0
1 ug/ml	3.56	101	3	97	0
0.3 ug/ml	3.53	101	9	91	0
0.1 ug/ml	3.49	99	8	92	0

[1]CHO cells were treated in the absence of an exogenous source of metabolic activation for 6 hours at 37±1°C.

[2]Relative Cell Growth = (Cells/treatment flask x 100)/Cells/solvent flask.

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TABLE 1a

PRELIMINARY TOXICITY TEST USING TECHNICAL DICAMBA IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

Treatment[1]	Growth Potential		Cell Cycle Kinetics		
	Cells/flask ($\times 10^6$)	Relative Cell Growth[2] (%)	Percentage of Cells in M_1	M_2	M_3
DMSO	3.77	100	5	95	0
Technical Dicamba 2560 ug/ml	4.04	107	3	97	0
2180 ug/ml	4.11	109	5	95	0
1830 ug/ml	4.18	111	6	94	0
1460 ug/ml	3.83	102	4	96	0
1100 ug/ml	3.82	101	4	96	0
740 ug/ml	3.91	104	3	97	0

[1]CHO cells were treated in the absence of an exogenous source of metabolic activation for 6 hours at 37±1°C.

[2]Relative Cell Growth = (Cells/treatment flask x 100)/Cells/solvent flask.

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TABLE 2

PRELIMINARY TOXICITY TEST USING TECHNICAL DICAMBA IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

Treatment[1]	Growth Potential		Cell Cycle Kinetics		
	Cells/flask ($\times 10^6$)	Relative Cell Growth[2] (%)	Percentage of Cells in M_1	M_2	M_3
DMSO	4.05	100	0	100	0
Technical Dicamba					
1000 ug/ml	4.55	112	5	95	0
300 ug/ml	3.99	99	6	94	0
100 ug/ml	4.57	113	6	94	0
30 ug/ml	3.77	93	5	95	0
10 ug/ml	4.38	108	6	94	0
3 ug/ml	4.06	100	4	96	0
1 ug/ml	3.88	96	8	92	0
0.3 ug/ml	3.60	89	1	99	0
0.1 ug/ml	3.85	95	5	95	0

[1]CHO cells were treated in the presence of an exogenous source of metabolic activation for 2 hours at 37±1°C.

[2]Relative Cell Growth = (Cells/treatment flask x 100)/Cells/solvent flask.

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TABLE 2a

PRELIMINARY TOXICITY TEST USING TECHNICAL DICAMBA IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

Treatment[1]	Growth Potential		Cell Cycle Kinetics		
	Cells/flask ($\times 10^6$)	Relative Cell Growth[2] (%)	Percentage of Cells in M_1	M_2	M_3
DMSO	4.17	100	2	98	0
Technical Dicamba 2560 ug/ml	3.99	96	11	89	0
2180 ug/ml	3.90	94	5	95	0
1830 ug/ml	3.97	95	4	96	0
1460 ug/ml	3.88	93	2	98	0
1100 ug/ml	4.00	96	5	95	0
740 ug/ml	3.92	94	4	96	0

[1]CHO cells were treated in the presence of an exogenous source of metabolic activation for 2 hours at 37±1°C.

[2]Relative Cell Growth = (Cells/treatment flask x 100)/Cells/solvent flask.

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TABLE 3

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CYTOGENETIC ANALYSIS OF CHO CELLS TREATED WITH TECHNICAL DICAMBA IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

Treatment[1]	Cells Scored	Number and Types of Aberrations Scored[2]								Cells with Structural Aberrations (%)[3]	Structural Aberrations Per Cell[3,4,5]
		G	TB	SB	D	R	EX	PU	SDC		
Untreated	100	1	0	0	0	0	0	0	0	0	0.00
DMSO	100	0	2	0	1	0	0	0	0	2	0.03
Technical Dicamba											
2330 ug/ml	100	3	2	0	1	0	0	0	0	2	0.03
1170 ug/ml	100	4	1	0	1	0	0	0	0	2	0.02
590 ug/ml	100	7	0	0	1	0	0	0	0	1	0.01
300 ug/ml	100	2	1	0	0	0	0	0	0	1	0.01
TEM											
1 ug/ml	100	3	21	0	1	0	3	1	1	20	0.45**

[1]CHO cells were treated for 8 hours at 37±1°C in the absence of an exogenous source of metabolic activation.

[2]G=chromosome and chromatid gaps; TB=chromatid breaks and fragments; SB=chromosome breaks and acentric fragments; D=dicentric; R=ring; EX=exchanges (includes quadriradials, triradials and complex rearrangements); PU=pulverization; SDC=cells with 10 or more structural aberrations.

[3]Chromatid and chromosome gaps are not included.

[4]*, $p \leq 0.05$; **, $p \leq 0.01$ (Student's t test).

[5]SDC and pulverizations were counted as 10 aberrations.

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TABLE 4

CYTOGENETIC ANALYSIS OF CHO CELLS TREATED WITH TECHNICAL DICAMBA IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

Treatment[1]	Cells Scored	Number and Types of Aberrations Scored[2]								Cells with Structural Aberrations (%)[3]	Structural Aberrations Per Cell[3,4,5]
		G	TB	SB	D	R	EX	PU	SDC		
Untreated	100	0	0	0	0	0	0	0	0	0	0.00
DMSO	100	4	0	0	3	0	0	0	0	3	0.03
Technical Dicamba											
2330 ug/ml	100	1	1	0	1	0	0	0	0	2	0.02
1170 ug/ml	100	2	2	0	1	0	0	0	0	3	0.03
590 ug/ml	100	3	1	0	0	0	0	0	0	1	0.01
300 ug/ml	100	2	1	0	0	0	0	0	0	1	0.01
CP											
100 ug/ml	100	2	10	2	1	2	14	1	3	22	0.69**

[1]CHO cells were treated for 2 hours at 37±1°C in the presence of an exogenous source of metabolic activation.

[2]G=chromosome and chromatid gaps; TB=chromatid breaks and fragments; SB=chromosome breaks and accentric fragments; D=dicentric; R=ring; EX=exchanges (includes quadriradials, triradials and complex rearrangements); PU=pulverization; SDC=cells with 10 or more structural aberrations.

[3]Chromatid and chromosome gaps are not included.

[4]*, $p \leq 0.05$; **, $p \leq 0.01$ (Student's t test).

[5]SDC and pulverizations were counted as 10 aberrations.

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